Journal of Medical Virology 60:337–341 (2000)

HPV16 E6 Oncogene Variants in Women With Cervical Intraepithelial Neoplasia

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Human papillomaviruses (HPVs) are strongly associated with the development of high grade cervical intraepithelial neoplasia (CIN) and cervical carcinoma, with between 40-80% of patients with cervical carcinoma being attributed to a single HPV type, HPV16 depending on the methods used and geographical location of the particular study [van den Brule et al., 1996]. An HPV16 E6 variant has been described which is strongly associated with high grade CIN [Ellis et al., 1997] and with the human leukocyte antigen (HLA)-B7 genotype in women with cervical carcinoma where HLA-B7 positive patients were demonstrated to have a significantly poorer clinical outcome [Ellis et al., 1995]. To determine whether this HPV16 E6 variant might play a significant role in the pathogenesis of cervical disease, 174 HPV16 positive women were selected from those attending the colposcopy clinics of Guy's and St Thomas' Hospital Trust following polymerase chain reaction (PCR) amplification of HPV16 L1 or E5 DNAs from cervical brush swabs or fixed biopsy tissue. HPV16 E6 DNA was amplified by PCR and the variant sequence was identified by Msp 1 restriction enzyme digestion, as the nucleotide substitution creates an additional unique Msp 1 site. The study group comprised 29 normal controls, 7 women with borderline cytology, 123 women with cervical dysplasia and 12 women with cervical cancer. 101/174 (58%) of these women had amplifiable E6 DNA and restriction enzyme digestion was performed on 95 of these. The variant E6 sequence was identified in 3/95 (3%) individuals, two of whom had normal histology and one had a CIN II lesion. Wild type E6 sequence was identified in the remaining 92/95 (97%) individuals. These data suggest that this particular E6 variant does not play a major role in the pathogenesis of HPV16 related cervical disease in women living in the South London area. ***J. Med. Virol. 60:337–341, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: HPV16 variants; human papillomavirus; cervical intraepithelial neoplasia; cervical carcinoma

INTRODUCTION

High grade cervical intraepithelial neoplasia lesions are those most likely to progress to cervical cancer and to be associated with a "high-risk" human papillomavirus type, most commonly type 16. Most CIN lesions regress naturally if left untreated demonstrating that most individuals are able to generate an effective HPV specific immune response. The fact that a small proportion of high grade CIN lesions progress to cervical carcinoma demonstrates that HPV can evade the host immune response in some cases. We are interested in identifying mechanisms by which this might occur having shown that most women with CIN lesions are able to mount peripheral blood T-helper cell responses to the HPV16 major capsid protein L1 but that T-cell responses to the E7 oncoprotein are downregulated in comparison to healthy individuals [Luxton et al., 1996; Shepherd et al., 1996]. Non prototype variants of HPV16 have been shown to confer a greater risk of development of CIN grade II/III lesions when compared to prototype [Xi et al., 1997]. In a few studies this type of sequence variation has been postulated to lead to evasion of host immune recognition. Ellis et al. [1995] described an HPV16 variant with a single

Grant sponsor: Cancer Research Campaign; Grant sponsor: The Special Trustees of Guy's and St. Thomas' Hospital.

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Accepted 21 July 1999

nucleotide substitution (adenosine for guanine) at position 131 of the E6 oncogene which was originally found in a group of HLA-B7 positive women with cervical carcinoma. The HLA-B7 genotype was found to be associated with a poorer clinical outcome in these patients. It was suggested that both the down-regulation of HLA-B7 seen on tumour cells and the association of HLA-B7 with the variant strain of HPV16 might contribute to evasion of the host immune response by the virus. A subsequent study conducted in Birmingham, UK found that 30% (29/95) of women investigated for persistent low grade cervical cytological abnormalities were infected with the variant strain [Ellis et al., 1997]. Infection with the variant was significantly associated with underlying high grade CIN and a reduced ability to produce serum antibodies against the HPV16 major capsid protein L1 when compared to women infected with wild type virus. The mechanisms by which L1 specific antibody production were reduced were not further investigated therefore it is not known whether they are a direct result of the E6 nucleotide variation or a reflection of further variation in the L1 gene. However, the association of the variant with high grade CIN strongly suggests that this virus is able to evade immune recognition and as such may be important in the pathogenesis of cervical disease. It was from this viewpoint that we studied a group of women with abnormal cervical cytology receiving treatment at Guy's and St Thomas' Hospitals to determine whether this variant plays a significant role in the pathogenesis of their disease.

METHODS

Selection of Patients and Controls

The patients studied were those attending the colposcopy and well women's clinics of Guy's and St Thomas' Hospital Trust for investigation of abnormal smears. Cervical biopsies were taken by the investigating physician where necessary for the purpose of diagnosis otherwise ethical approval was obtained to take a cervical brush swab sample for HPV detection. 174 HPV16 DNA positive women were selected for further study on the basis of a positive HPV16 polymerase chain reaction (PCR) performed on cervical biopsy tissue sections or cells from a cervical brush as described below. Clinical diagnoses made on the basis of histological evidence from cervical biopsies indicated that 29 individuals had normal histology, 28 CIN I lesions, 43 CIN II lesions and 55 CIN III lesions. Seven women had borderline cytology (no biopsies were taken).

Sample Preparation

(i) Paraffin fixed cervical biopsies. 10 μm tissue sections were cut, dewaxed, proteinase K digested and stored at −20°C prior to PCR as we have previously described [Shepherd et al., 1996]. (ii) Cervical brush swabs. These were collected into 5ml sterile PBS and stored at −20°C before further processing. Samples were vortexed vigorously for 5 min to release cell debris from the brush which was then discarded. Samples were centrifuged at 1000 g for 10 min to pellet cellular material. The cell pellet was then digested using proteinase K as above.

Polymerase Chain Reactions

(i) Screening for HPV 16 positivity. HPV 16 positive individuals were selected by PCR amplification of the L1 or E5 genes from cervical biopsy tissue or cervical brush swab material by established methods [van den Brule et al., 1992; Cavuslu et al., 1996]. (ii) HPV 16 E6 PCR. Reaction conditions were essentially as for L1 consensus PCR except that hot start PCR was carried out and the $MgCl_2$ concentration was 2.5 mM. Two different primer sets were used; E6.1A [GAGAACTGCAATGTTTCAGG] and E6.2A [TGATTACAGCTGGGTTTCTC] which amplify a 469bp fragment of E6 and E6.1B [CCAAAAGAGAACTGCAATGT] and E6.2B [AATTTTAGAATAAAACTTTAAACATT] which amplify a 234bp fragment of E6. PCR products were visualized on a 1.5% agarose gel.

E6 Variant Identification

E6 PCR products were first subjected to phenol-chloroform extraction. Variants were identified by their unique Msp 1 restriction site at nucleotide position 131 essentially as described by Ellis et al. [1995] except that the E6 PCR products differed in length (see Fig. 1). Hence, the 469bp product of amplification with set A primers was first digested with Nsi 1 to give fragments of 163 and 306bp. These products were digested further with Msp 1, cutting the 306bp fragment into 245 and 61bp fragments and cutting the 163bp into 127 and 36bp fragments only when variant DNA is present. The E6 PCR product, Nsi 1 and Msp 1 digests were run in adjacent wells of a 1.5% agarose gel where a change from a 163bp fragment in lane 2 to a 127bp fragment in lane 3 indicates presence of the variant and no change indicates presence of the wild type. The 234bp products of amplification with set B primers were simply digested with Msp 1 giving 192 and 42bp fragments in the presence of variant DNA only. Hence PCR product and Msp 1 digest were run in adjacent wells and a change from a 234bp fragment in lane 1 to a 192bp fragment in lane 2 indicates the presence of the variant and no change the wild type.

Restriction Enzyme Digestion

Following amplification with set A primers, 5 μl of phenol extracted E6 PCR product was digested with 10 units of Nsi 1 in a final volume of 20 μl digestion buffer (Boehringer Mannheim, Mannheim, Germany) at 37°C overnight. 10 μl of the product of Nsi 1 digestion was then digested with Msp 1 under the same conditions except that BSA was added to a final concentration of 0.2 mg/ml. PCR products from amplification with primer set B were digested with Msp 1 only as above.

RESULTS

One hundred and seventy-four women were selected for study following positive PCRs for HPV16 L1 or E5

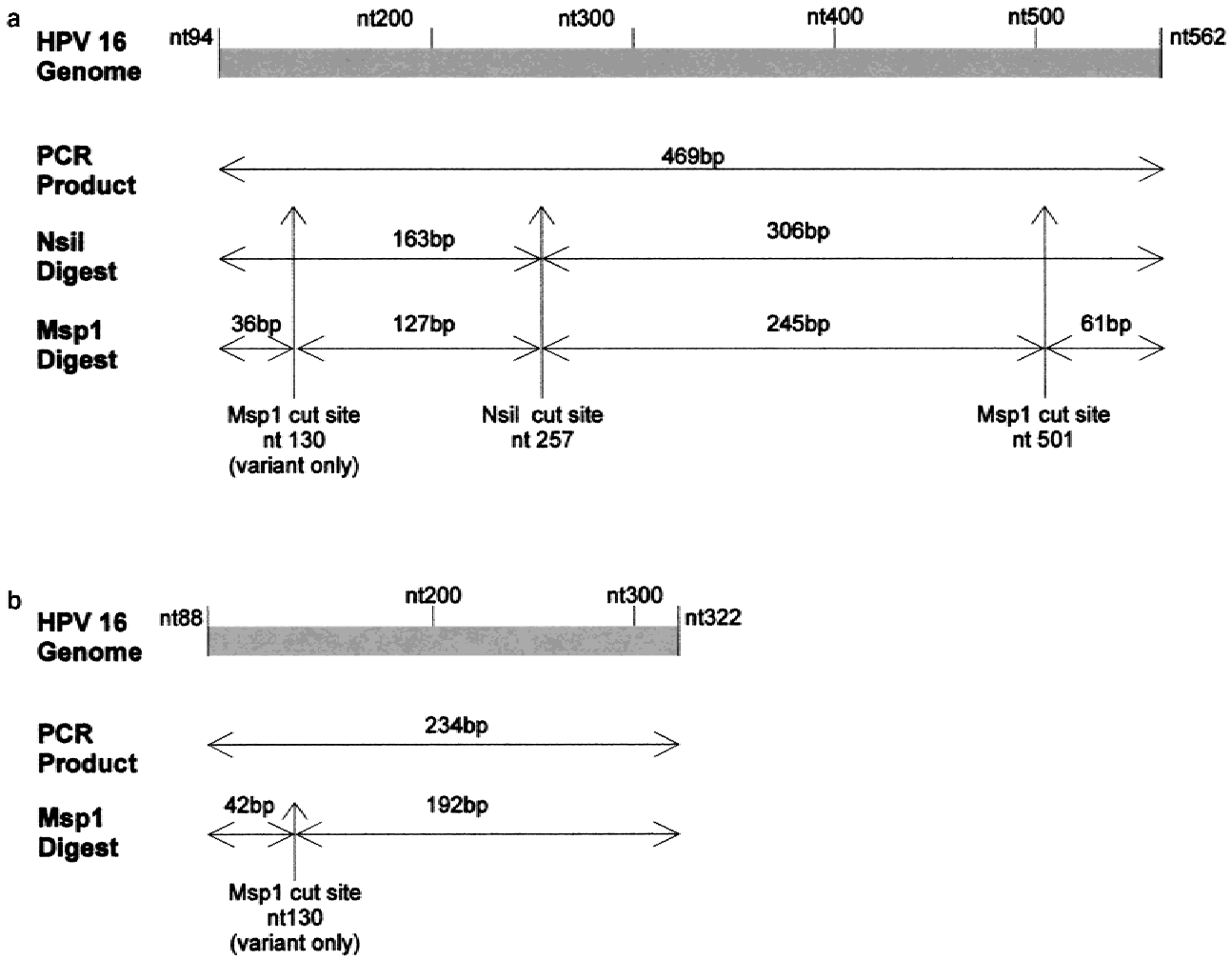

Fig. 1. Restriction enzyme digest analysis of products of E6 PCR using **(a)** primer set A, **(b)** primer set B. Enzyme cut sites are present in both WT and variant DNA unless otherwise indicated.

TABLE I. HPV16 Typing and E6 Variant Analysis Performed on DNA Extracts From Patients With Cervical Dysplasia or Cervical Cancer and Normal Controls

	Normal	Borderline cytology	CIN I	CIN II	CIN III	Cervical cancer	Total
HPV 16 DNA positive	29	7	28	43	55	12	174
HPV 16 E6 positive	15	4	19	26	31	6	101
biopsies	13	4	18	14	22	4	75
brush swabs	2	0	1	12	9	2	26
E6 variant	2*	0	0	1**	0	0	3
E6 wild type	13	4	18	20	31	6	92

*One variant was found in a fixed biopsy sample and the other in a brush swab.
**The variant was found in a fixed biopsy sample.

DNA undertaken on DNA extracts from paraffin embedded cervical biopsies or from cervical brush swab samples. Data obtained from these patients are summarised in Table I. Clinical diagnoses based on histological evidence from cervical biopsies showed that the group included 29 women with normal histology, 123 women with cervical dysplasia (including 28 with CIN I, 43 with CIN II and 55 with CIN III lesions) and 12 women with cervical cancer. Seven women were classified as borderline cytology (no biopsies were taken in these cases). E6 DNA was amplified from 101/174 (58%) of patients' DNA extracts, (where 75 were from cervical biopsies and 26 from brush swabs). In a direct comparison of PCRs performed on DNA extracts from paraffin-embedded formalin fixed tissue (data not shown) the primers which amplified the shorter E6 sequence, the E6B primers, were found to be more sensitive than the E6A primers, amplifying the E6 gene in

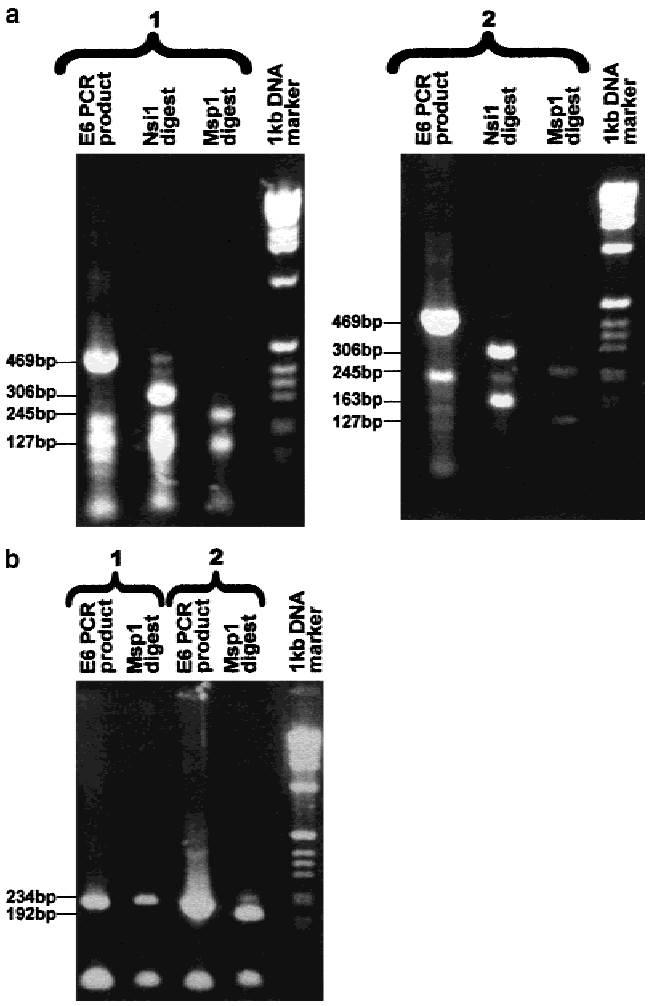

Fig. 2. Identification of E6 variants by restriction enzyme digestion of E6 PCR products **(a)** using PCR primer set A and **(b)** using PCR primer set B. 1; examples of wild type isolates and 2; variant isolates. The size of PCR and the major visible restriction digest products are labeled. (a) E6 PCR product is 469bp, cut by Nsi1 to give 306bp+163bp products and Msp1 to give 245bp+163bp products in the wild type and 245bp+127bp products in the variant. (b) E6 PCR product is 234bp, Msp1 digestion does not cut the wild type DNA but cuts variant DNA to give a 192 bp visible fragment.

44% compared to 24% of HPV16 positive DNA extracts. It is considered that this is a reflection of a failure to amplify larger fragments due to DNA fragmentation often seen in formalin fixed tissue samples. Sufficient E6 PCR product was obtained to perform restriction enzyme digestions on samples from 95 individuals. Examples of variant and wild type DNA identified by restriction enzyme digestion of the PCR products of both E6 primer sets are shown in Figure 2. Msp 1 digestion identified the variant E6 sequence in 3/95 (3%) individuals, two of whom had normal histology and one had a CIN II lesion. Wild type E6 sequence was identified in the remaining 92/95 (97%) individuals. No association of this variant with high grade dysplasia, which constituted ⅓ of our patient group was therefore found. There was no association of the variant with cervical cancer although the number of E6 positive cervical carcinomas studied was small.

DISCUSSION

In conclusion, this particular HPV16 E6 variant does not appear to play a major role in the pathogenesis of HPV16 related cervical disease in women from the South East of England who are treated at Guy's and St Thomas' Hospitals. However, as this is a cross-sectional study we cannot rule out the possibility that women carrying this variant may still be more susceptible to the development of high grade cervical lesions or indeed cervical carcinoma. Prospective studies will be required to answer this question. Interestingly, on further investigation of the previous medical histories of these patients, one of those carrying the variant, who currently had normal cervical histology, was found to have had previous treatment for a CIN III lesion. The results reported clearly differ significantly from those of Ellis et al. [1997] who reported a 30% incidence of the variant amongst women with persistent cervical cytological abnormalities compared to only 3% in our study group. This variant was originally shown to be strongly associated with HLA-B7 [Ellis et al., 1995]. However, in a recent study of 241 women with minor cervical cytological abnormalities no association with any HLA type was found even though a high percentage of patients (20.3% of all patients or 28% of HPV16 positive cases) were found to be carrying the variant virus [Etherington et al., 1999]. There is no clear explanation as to why this variant is considerably more prevalent in some studies than in others. In the absence of an association with HLA it is important to identify other factors that may be associated with acquisition of this variant. The prevalence of such variants is likely to be affected by geographical location and therefore the ethnic origin of the patients studied may be an important factor. Also, although numerous HPV16 variants have been described, mechanisms by which host immune recognition might be evaded have only been investigated in a few cases. The resolution of these issues will be important in order to ensure that broadly effective HPV vaccines are developed.

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